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An Intersectional Hybrid in the Genus *Pteris* (Pteridaceae) on Okinawa Island: Evidence from Nuclear and Chloroplast DNA

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A putative hybrid of the fern genus *Pteris* was collected on Okinawa Island. It was morphologically intermediate between *P. ryukyuensis* (section *Pteris*) and *P. semipinnata* (section *Campteria*), suggesting that it might be an intersectional hybrid between them. The hybrid nature of the plant also suspected because of the abnormal morphology of the spores and sporangia. To clarify the parentage of this putative hybrid, we analyzed the nuclear (nDNA) and chloroplast (cpDNA) DNA of all three of them. PCR analysis of the internal transcribed spacer 1 region of the nDNA indicated that the putative hybrid possessed nDNA of *P. ryukyuensis*. Furthermore, PCR-SSCP analysis of the intergenic spacer between *psbC* and *trnS* of cpDNA showed the putative hybrid to have the same electrophoretic pattern as *P. semipinnata*. Because the cpDNA is generally maternally inherited, we concluded that the putative hybrid was an intersectional hybrid between *P. ryukyuensis* and *P. semipinnata*, with the latter species functioning as the mother.

Key words: internal transcribed spacer, intersectional hybrid, natural hybrid, Okinawa Island, PCR-SSCP, *Pteris ryukyuensis*, *Pteris semipinnata*, ribosomal DNA

Pteris L. is a genus of ferns with more than 250 species distributed in the tropics and subtropics worldwide (Iwatsuki 1992). Shieh (1966) recognized 37 taxa of *Pteris* in Japan and Taiwan and classified them into two subgenera and four sections. Many species of the genus *Pteris* are distinctive and somewhat isolated, but natural hybridization events have been reported for various combinations of taxa (e.g., Walker 1957, Nakato 1975, Suzuki & Iwatsuki 1990, Iwatsuki 1992). The known hybrids of *Pteris*, however, have arisen through crosses between closely related taxa and intersectional hybrids have rarely been reported.

In 1999, Mr. Gen-ei Hokama found unusual plants of *Pteris* on Okinawa Island. They were growing together with two species of *Pteris*: *P. ryukyuensis* Tagawa (section *Pteris*) and *P. semipinnata* L. (section *Campteria*). Those two species are morphologically distinct: *P. ryukyuensis* has dimorphic, trifoliate or simply pinnatifid fronds with one or two pairs of lateral pinnae; *P. semipinnata* has monomorphic fronds with one to five pairs of lateral pinnae and with the lower pinnae of the fronds lobed only on the outer side. Although the gross morphology of the unusual plant collected by Mr. Hokama is somewhat similar to that of *P. ryukyuensis* in its dimorphic fronds, the fertile and sterile fronds have three to five pairs of lateral pinnae, and the lower pinnae of the fertile fronds have lobes on the outer side, somewhat similar to *P.*

semipinnata. Based on these morphological characteristics, we suspected that the unusual plant was a natural intersectional hybrid of the sympatric *P. ryukyuensis* and *P. semipinnata*. To confirm our suspicions, we examined the spore morphology of the putative hybrid. We further analyzed the nuclear and chloroplast DNA to clarify whether *P. ryukyuensis* and *P. semipinnata* are both involved in the origin of this putative hybrid.

Materials and Methods

The putative hybrid was collected by Mr. Gen-ei Hokama in Uchikina, Okinawa City, Okinawa Prefecture. Additionally, eight of the nine species of *Pteris* on Okinawa Island were collected for comparison, including four species (*P. ensiformis* Burmann, *P. nipponica* Shieh, *P. ryukyuensis* and *P. vittata* L.) of section *Pteris*, three species (*P. semipinnata*, *P. dispar* Kunze and *P. fauriei* Hieronymus) of section *Campteria*, and one species (*P. wallichiana* Agardh) of section *Tripedipteris* (Table 1). The infrageneric grouping of these taxa follows Shieh (1966). Each of the putative parents, *P. ryukyuensis* and *P. semipinnata*, was collected at the two localities shown in Table 1. Living plants were transplanted to the greenhouse of the University of the Ryukyus and voucher specimens were deposited in the herbarium of the Faculty of Science, University of the Ryukyus (RYU). The spore morphology of the putative hybrid and of the two putative parents, *P. ryukyuensis* and *P. semipinnata*, was observed. Spores collected from dried specimens were mounted on glass slides and stained following the procedure of Alexander (1980). The morphology of the spores was examined with a light microscope.

All of the collections were used in the following molecular analysis. Total DNAs were prepared from fresh leaves of cultivated plants by the CTAB extraction method (Doyle & Doyle 1987). The internal transcribed spacer 1 (ITS1) of the nuclear ribosomal DNA was amplified

TABLE 1. Collection localities of the putative hybrid and eight taxa of the genus *Pteris* investigated in the present study. Intrageneric classification follows Shieh (1966).

Taxa		Collection localities
1. Putative hybrid		Uchikina, Okinawa city, Okinawa
2. Other *Pteris* species		
Subgenus *Pteris*		
Section *Pteris*	*P. ensiformis*	Taminato, Ogimi village, Okinawa
	P. nipponica	Oshikawa, Ogimi village, Okinawa
	P. ryukyuensis	Mashiki, Ginowan city, Okinawa
		Senbaru, Nishihara town, Okinawa
	P. vittata	Mashiki, Ginowan city, Okinawa
Section *Campteria*	*P. dispar*	Senbaru, Nishihara town, Okinawa
	P. semipinnata	Chibana, Okinawa city, Okinawa
		Oshittai, Nago city, Okinawa
	P. fauriei	Taminato, Ogimi village, Okinawa
Subgenus *Tripedipteris*		
Section *Tripedipteris*	*P. wallichiana*	Mt. Yonaha-dake, Kunigami village, Okinawa

by PCR using two primers (5'-CGTAAC AAGGTTTCCGTAG-3', 5'-ATCCGTTGC CGAGAGTCGT-3') designed by Maggini *et al.* (1998). The sequences of these primers are homologous to portions of 18S and 5.8S ribosomal DNA, respectively. The PCR mixture contained the following reagents: 66 μl of distilled H_2O; 10 μl of 10 x PCR buffer; 10 μl of 2 mM dNTPs; 8 μl of 25 mM $MgCl_2$; 2 μl of 4 pM solution of each primer; 1 μl of Taq DNA polymerase (TOYOBO); and 1 μl of total DNA template. The thermal cycle consisted of 30 cycles of 1 min at 94°C, 1 min at 45°C, and 2 min at 72°C. PCR products of the ITS1 region were cleaned using a QIAquick PCR Purification Kit (QIAGEN) following the manufacturer's recommendations. The PCR products were electrophoresed and separated on 4 % polyacrylamide gel. Gels were stained with a DNA silver staining kit (ATTO) and the length variations of the ITS1 region were detected and compared.

We performed the polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis of the intergenic spacer between *psbC* and *trnS* (UGA) of cpDNA. Hereafter, the analyzed region is simply designated as the *psbC-trnS* region. The *psbC-trnS* region was amplified by PCR using two primers (psbC-trnS/F and psbC-trnS/R) designed by Nishizawa & Watano (2000). Composition of the PCR mixture is the same as given for the ITS1 region described above. The thermal cycle consisted of 25 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. The PCR products of the *psbC-trnS* region were then cleaned and concentrated using a QIAquick PCR Purification Kit (QIAGEN). A portion of the purified PCR product (3 μl) was mixed with 17 μl of 95% formamide, 0.005% bromophenol blue, 0.005% xyren cyanol, and then denatured by heating at 85°C for 5 min. Denatured samples were immediately cooled on ice and electrophoresed on 6 % polyacrylamide gel (16 x 16 x 0.1 cm) in 0.5 x TBE buffer at 200V for 6 hr. After electrophoresis, gels were stained with DNA silver staining kit (ATTO) and SSCPs were detected.

Results and Discussion

Spores of the putative parents, *P. ryukyuensis* and *P. semipinnata*, were normal, homogenous and tetrahedral (Fig. 1a, b). Spores of the putative hybrid were conspicuously variable in shape and size (Fig. 1c, d). We did not find normal tetrahedral spores like those of the suspected parents in the putative hybrid. Nearly all the sporangia of the putative hybrid were aborted (Fig. 1e). These results indicate that the spores of the putative hybrid are sterile, and therefore support its hybrid nature.

The number and length of the PCR products of ITS1 region were heterogeneous among the nine taxa of *Pteris* analyzed (Fig. 2). The PCR products of *P. ryukyuensis* (Fig. 2, lane 3) consisted of three bands (ca. 400, 380, and 350 bp), of which the 350 bp band was diagnostic of this species, while those of *P. semipinnata* (Fig. 2, lane 6) consisted of two bands (ca. 380 and 335 bp). The 335 bp band of *P. semipinnata* also was found in *P. dispar* (section *Campteria*) (Fig. 2, lane 7). The putative hybrid (Fig. 2, lane 5) exhibited all the bands (ca. 400, 380, 350, and 335 bp) found in *P. ryukyuensis* and *P. semipinnata*. The 350 bp band is diagnostic of *P. ryukyuensis*, indicating that the putative hybrid has incorporated the nDNA of *P. ryukyuensis*, thus providing evidence that *P. ryukyuensis* (section *Pteris*) is one of the parents. The 335 bp band in the putative hybrid is shared not only with *P. semipinnata* but also with *P. dispar*, indicating that either *P. semipinnata* or *P. dispar* (section

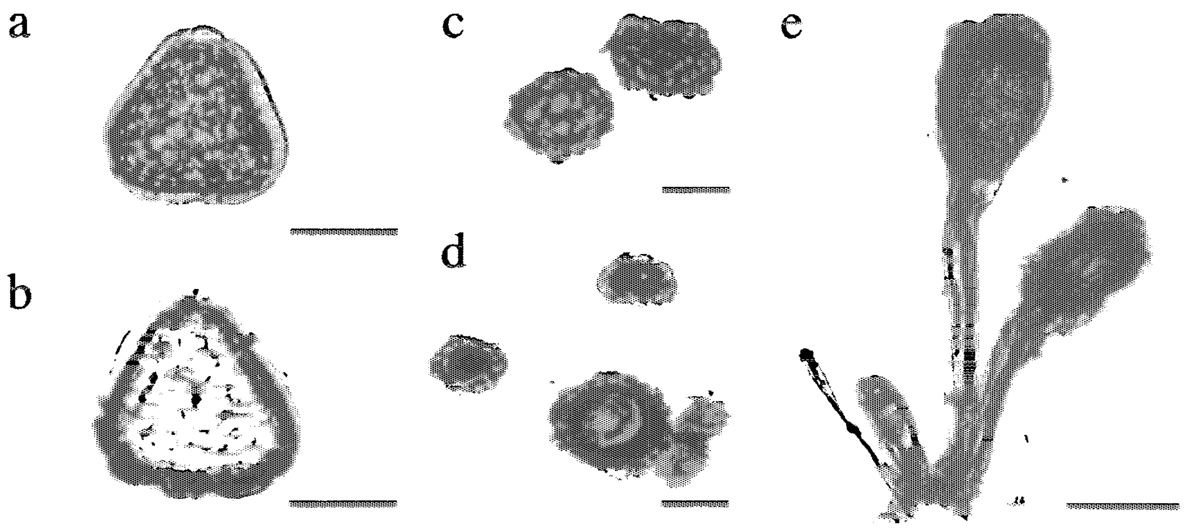


FIG. 1. Normal tetrahedral spores of *P. ryukyuensis* (a) and *P. semipinnata* (b); abnormal spores (c, d) and aborted sporangia (e) of the putative hybrid. Scales = 20 μm in a-d, 100 μm in e.

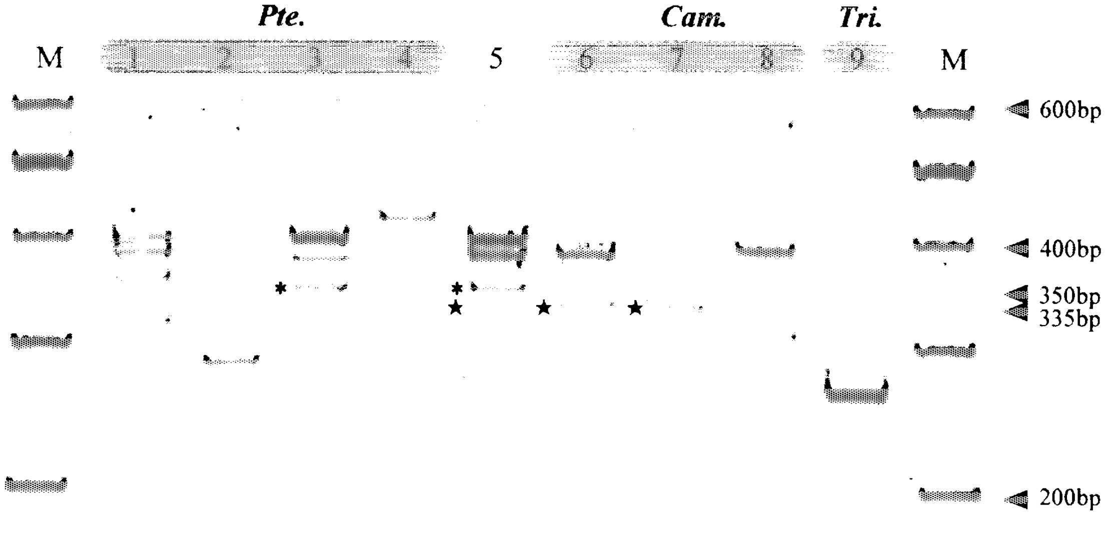


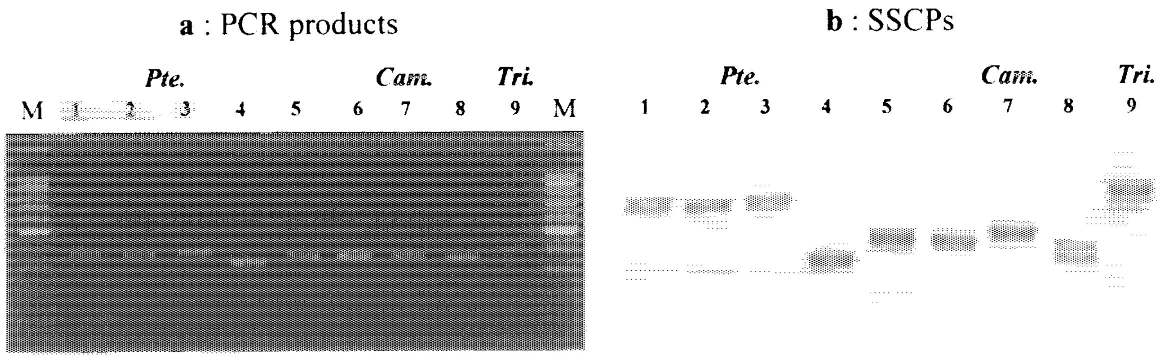
FIG. 2. PCR products of ITS1 region of nDNA electrophoresed on 4% polyacrylamide gel. Lane 1: *Pteris nipponica*; lane 2: *P. ensiformis*; lane 3: *P. ryukyuensis*; lane 4: *P. vittata*; lane 5: putative hybrid; lane 6: *P. semipinnata*; lane 7: *P. dispar*; lane 8: *P. fauriei*; lane 9: *P. wallichiana*. Asterisks and stars indicate respectively bands diagnostic of *P. ryukyuensis*, and *P. semipinnata* or *P. dispar*. Sectional grouping is indicated above lane number: *Pte*. section *Pteris*; *Cam*. section *Campteria*; *Tri*. section *Tripedipteris*.

Campteria) is the other parent. Because neither *P. semipinnata* nor *P. dispar* possess a species specific band, we cannot verify the second parent based on a comparison of the length of the ITS1 region. In contrast, the five remaining species, *P. nipponica* (ca. 360 and 320 bp), *P. ensiformis* (290 and 200 bp), *P. vittata* (420 bp), *P. fauriei* (580 and 310 bp), and *P. wallichiana* (270 bp)

(Fig. 2, lanes 1, 2, 4, 8, 9), have species specific bands in the ITS1 region. Since none of the species specific bands of those five species appeared in the putative hybrid, it is unlikely that *P. nipponica*, *P. ensiformis*, *P. vittata*, *P. fauriei* or *P. wallichiana* were involved in the parentage of the putative hybrid.

Although the ITS region is generally considered to be homogeneous (e.g., Baldwin *et al.* 1995), more than two different sequences of the ITS1 region are maintained in the analyzed taxa, with two exceptions: *P. dispar* and *P. wallichiana*. Each of these two species has only one sequence for the ITS1 region (Fig. 2, lanes 7, 9). Heterogeneity of the ITS region within individual plants has been reported in several plant taxa (e.g., Kim & Jansen 1994, Campbell *et al.* 1997). In *Tulipa*, the occurrence of a large number of ribosomal DNA (rDNA) loci might explain the heterogeneity in ITS region (Booy *et al.* 2000). Of the taxa of *Pteris* analyzed, both *P. dispar* and *P. wallichiana* are diploid, while the other six species investigated are triploids or tetraploids based on x=29 (Walker 1961, Mitui 1966, 1967, Kawakami 1971). Only the triploid and tetraploid taxa contain more than two different ITS sequences. We have no information about the rDNA loci in the taxa of *Pteris*, but it seems likely that several rDNA loci occur in the polyploid taxa of *Pteris*, causing the conspicuous heterogeneity in the number and length of the PCR products of ITS1 region.

CpDNA analysis provided further information about the direction of intersectional hybridization. All PCR products of the *psbC-trnS* region were ca. 350 bp in length, with two exceptions: *P. vittata* and *P. wallichiana* had PCR products of ca. 300 bp and 400 bp in length, respectively (Fig. 3a). The PCR-SSCPs of the *psbC-trnS* region are indicated in Fig. 3b. Only *P. semipinnata* showed the same single banded pattern as the putative hybrid (Fig. 3b, lanes 5, 6). Because cpDNA is generally maternally inherited, P. *semipinnata* (section *Campteria*) must have functioned as the maternal parent in the origin of the putative hybrid. *Pteris dispar* exhibits a slower single banded pattern than in the putative hybrid, and was rejected as a possible parent of the putative hybrid (Fig. 3b, lane 7). Band patterns of five other species were also distinct from those of the putative hybrid and from *P. semipinnata* because of their mobility and/or band number. *Pteris nipponica*, *P. ensiformis*, and *P. ryukyuensis* (section *Pteris*) (Fig. 3b, lanes 1, 2, 3) showed slower single banded or two banded patterns than those of the putative hybrid; *P. fauriei*


a : PCR products

b : SSCPs

FIG. 3. PCR products of *psbC-trnS* region of cpDNA (a) and their SSCPs (b). Lane 1: *Pteris nipponica*; lane 2: *P. ensiformis*; lane 3: *P. ryukyuensis*; lane 4: *P. vittata*; lane 5: putative hybrid; lane 6: *P. semipinnata*; lane 7: *P. dispar*; lane 8: *P. fauriei*; lane 9: *P. wallichiana*. Sectional grouping is indicated above lane numbers. *Pte.* section *Pteris*; *Cam.* section *Campteria*; *Tri.* section *Tripedipteris*.

(section *Campteria*) (Fig. 3b, lane 8) showed a faster two banded pattern than did the putative hybrid. *Pteris vittata* (section *Pteris*) (Fig. 3b, lane 4) and *P. wallichiana* (section *Tripedipteris*) (Fig. 3b, lane 9) showed the fastest and slowest single banded pattern, respectively, although the variation might have been due to length differences of the PCR products and not the actual SSCPs.

The results indicate that the putative hybrid is an intersectional cross between *P. ryukyuensis* (section *Pteris*) and *P. semipinnata* (section *Campteria*), with latter species as the maternal parent (Fig. 4). Although many hybrid taxa are known in *Pteris*, intersectional hybrids have rarely been reported (e.g., Walker 1957). Because *P. ryukyuensis* commonly grows in alkaline soils in open places and on limestone walls and *P. semipinnata* prefers to grow in acidic soils in moist, more or less shaded habitats, they are ecologically isolated to some extent. *Pteris ryukyuensis*, however, occasionally comes into contact with *P. semipinnata* in the central part of Okinawa Island where alkaline and acidic soils occur side by side, giving rise to the rare opportunity for intersectional hybrids to be produced. Natural hybridization is recognized as a significant evolutionary force leading to the formation of new taxa in *Pteris* (e.g., Walker 1957, Suzuki & Iwatsuki 1990). The existence of intersectional hybrids may further increase genetic variability and lead to new taxa with novel genetic combinations in *Pteris*. To elucidate the ecological and evolutionary significance of intersectional hybrids in *Pteris*, further studies of the cytology and breeding system of the hybrid are needed.

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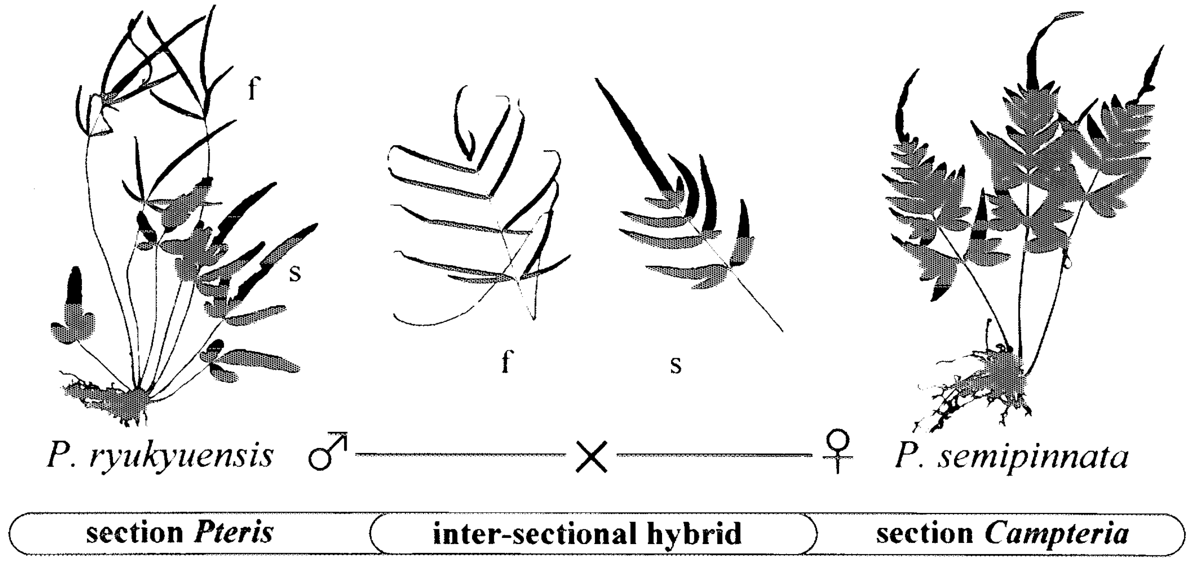

FIG. 4. Diagram indicating relationship of intersectional hybrid and parental taxa, *P. ryukyuensis* (section *Pteris*) and *P. semipinnata* (section *Campteria*). *Pteris semipinnata* as maternal parent in hybridization process. f : fertile frond. s : sterile frond.

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